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(57) Abstract

The present invention relates to a high density tethered polymer surface material comprising two regions: (1) a polymer shell characterized by an optimal surface concentration of aldehyde, hydroxyl or sulfhydryl groups; and (2) polymer chains tethered to the shell via the surface groups. The chains function to exclude biomolecules and polymers from approaching the polymer core, thereby minimizing adsorption of such molecules to the surface material. The method of synthesizing this surface involves initiating polymerization of the surface polymer chains from the surface groups using CeIV, which optimizes the density of chains tethered to the surface. The surface concentration of aldehyde or sulfhydryl groups determines the chain density, and the composition of the copolymer constituting the shell determines the surface concentration of the aldehyde or sulfhydryl groups. If the polymer shell is coated onto a core particle, this surface has use as a size exclusion medium for chromatography. When coated onto other structures, this surface has application as a biocompatible material, because of the resulting exclusion qualities of the relatively high polymer chain density.

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A TETHERED POLYMER MACROMOLECULE-EXCLUDING SURFACE, ITS MODE OF SYNTHESIS AND USE

FIELD OF THE INVENTION

This invention relates to a biomolecule-excluding polymer surface for use as a biocompatible material or for size exclusion chromatography.

BACKGROUND OF THE INVENTION

Surface and interfacial chemistry concerns the processes that occur at the boundary between gas- liquid, liquid-liquid, liquid-solid, or gas-solid interfaces. The chemistry and physics at surfaces and interfaces govern a wide variety of technologically significant processes, including biocompatible materials, where schemes to reduce adhesion of biomolecules such as protein and calcium depositions, while enhancing tissue integration, are critical to the implantation of prosthetic devices. Likewise, this area of chemistry underlies the separation of molecules using chromatographic techniques.

Chromatography entails a separation method whereby individual chemical compounds which were originally present in a mixture are resolved from each other by the selective process of distribution between two heterogeneous phases. The distribution of chemical species to be separated occurs in a dynamic process between the mobile phase and the stationary phase.

The stationary phase is a dispersed medium, which usually has a relatively large

surface area, through which the mobile phase is allowed to flow. The chemical nature of the stationary phase exercises the primary control over the separation process. The greater the affinity of a particular chemical compound for the stationary medium, the longer it will be retained in the system. In other terms, the adsorptive effect of the chromatographic medium for different solutes determines their rates of migration through the medium. Exclusion of a compound will result in a rapid passage through the chromatography medium.

The phenomenon of adsorption, which is a basic thermodynamic property of interfaces, resulting from a discontinuity in intermolecular or interatomic forces, is important in nearly all industrial processes and products. Not only is adsorption the basic phenomenon of chromatographic separations, but is a key process that underlies the use of soaps, wetting agents, lubricants and surface treatments.

One area for which exclusion phenomena plays a foundational role is gel permeation chromatography, wherein the size separation of macromolecules has become a standard method for the separation of biopolymers, in particular of proteins and nucleic acid sequences.

Gel exclusion chromatography is associated with the equilibrium behavior of macromolecules interacting with the gel material, that is, with the partition of a macromolecule between the stationary and mobile phases (Giddings et al., 1968). Hence, the migration rate of a particular species down a column is directly related to its partition coefficient between the gel and the surrounding medium. Thermodynamic theories relevant to exclusion chromatography therefore center on calculation of this partition coefficient.

Gel permeation chromatography requires support materials possessing a hydrophilic surface and which have if possible no unspecific adsorption behavior. To avoid the

strongly unspecified adsorption behavior which occurs when underivatized porous silica gels are used, U.S. Patent No. 5,035,803 proposes that the surfaces of the pores in the silica gel be occupied by water-soluble vinyl polymers. The grafting process used in U.S. Patent No. 5,035,803 provides polymers which are randomly connected to the base support at any point of the polymer chain.

It is generally believed that the pore width of the chromatographic support material has to be matched to the respective separation problem. The processes which are used for setting the pore width of separation materials frequently require a great deal of effort. For this purpose, either the degree of cross-linking in the polymerization is adjusted or the pores of silica gel are widened by post-treatment steps.

It has been found, however, that wide-pored support materials whose pore width is so great that no separation or only insufficient separation of substances is now possible on the basis of gel permeation chromatography give excellent separation results if linear polymers of water-soluble vinyl monomers are grafted on to the aliphatic hydroxyl groups of these supports. In these support materials, one terminal monomer unit is in each case covalently bonded to the base support.

Another gel permeation material, described in WO 94/26379, combines the ideas of grafted polymers with wide-pore support materials. This material allows substances in a mixture to be separated on support material comprising linear polymers of water-soluble vinyl monomers which are grafted onto aliphatic hydroxyl groups of the base support and which are covalently bonded by a terminal monomer unit to the base support. The separation method of this invention uses a wide-pored matrix whose pore space is completely accessible to the analyte. In the chromatographic separation method using this support material, the diffusion of macromolecules is as strongly influenced by the linear polymers grafted onto the base support as is similarly known from separations in the gel permeation chromatography of the prior art.

These supports and their methods demonstrate the general theory regarding gel exclusion chromatography for which, in most calculations of the partition coefficient, the stationary phase is considered to be "porous". The ratio of the equilibrium concentration of the distributing species inside the gel to that in the bathing medium is the required quantity. It is generally calculated by assuming pores of various shapes or size distributions to characterize the gel (Porath, 1963; Laurent and Killander, 1964). The geometric limitations suffered by the distributing species attempting to occupy the pores produce a geometry-dependent reduction in concentration inside the gel that defines the partition coefficient (Porath, 1963; Laurent and Killander, 1964; Giddings et al., 1968). The results are qualitatively in agreement with the observation that larger molecules are excluded more than are smaller molecules, but the molecular property of the distributed material which should correlate best with chromatographic behavior is less clear (Giddings et al., 1968).

United States Patent No. 5,585,236 describes the separation of nucleic acids on nonporous polymer beads having an average diameter of about 1-100 microns, and which are suitable for chromatographic separation of mixtures of nucleic acids when the polymer beads are alkylated with alkyl chains having at least three carbon atoms. This procedure is based upon adsorption chromatography, for which an elution profile will be generated wherein the smaller molecules elute first and the larger molecules elute last. The separation is accomplished within a gradient that causes the small fragments to elute in front of the larger ones.

It is important to note that this type of adsorption chromatography elution profile is opposite to that obtained for gel exclusion chromatography. In the latter method, the larger molecules are excluded from the surface and thus pass over the surface, eluting before the smaller molecules which travel travel the additional distance created by pores or some matrix-like material such as polyacrylamide gel.

United States Patent Number 5,453,186 and Canadian Patent 1,330,074 describe polymeric separating materials, however these materials are based on supports containing hydroxyl groups. These types of surface initiation sites are not optimal for polymerization because the rate of polymerization off of hydroxyl groups proceeds too fast to generate the density and quality of polymeric tethers necessary for high quality size exclusion separation materials. Thus, a need still remains in the art for high quality size exclusion separation materials.

Another area where adsorption of biomolecules on a synthetic surface is of prime importance is the field of biomaterials science. In fact, the implantation of such biomaterial articles as substitute blood vessels, synthetic and intraocular lenses, electrodes, catheters and the like in and onto the body is a rapidly developing area of medicine. A primary impediment to the long-term use of such biomaterial implantables as synthetic vascular grafts has been the lack of satisfactory graft surfaces. The uncoated surfaces of synthetic blood vessels made from plastics, for example, often stimulate rapid thrombogenic action. Various plasma proteins play a role in initiating platelet and fibrin deposition on plastic surfaces. These actions lead to vascular constriction to hinder blood flow, and the inflammatory reaction that follows can lead to the loss of function of the synthetic implantable.

It is widely accepted that the biocompatibility of materials depends largely on their surface properties and the reactions which occur when the material comes in contact with the biological milieu. These reactions are understood to varying degrees. The most intense effort has been expended in studying biomaterial/blood interactions (Brash, J. L. and Horbett, T. A. (Eds), (1987) Proteins at Interfaces: Physicochemical and Biochemical Studies, American Chemical Society, Washington, 1) but many other areas have received attention, including restorative dental treatment (Glantz, P.-O. J., Attström, R.W., Meyer, A. E. and Baier, R. E. (1991) In: Interfacial

Phenomena in Biological Systems, M. Bender (Ed), Marcel Dekker, Inc., New York, 77), soft tissue implants (Gristina, A. G., Myrvik, Q. N., Naylor, P.T. and Meandor, T. L. (1991) In: Interfacial Phenomena in Biological Systems, M. Bender (Ed), Marcel Dekker, Inc., New York, 105) and tissue culture cell compatibility (Crooks, C. A., Douglas, J. A., Broughton, R. L. and Sefton, M. V. (1990) J. Biomed. Mat. Res. 24:1241). Protein adsorption from plasma or lymph is a primary event and most investigators in the field believe that the subsequent fate of foreign material follows directly from the nature of this adsorption. For instance, whole blood rapidly clots upon exposure to most non- biological interfaces due to the surface activation of Factor XII (Ratnoff, O. D. (1971) In: Thrombosis and Bleeding Disorders: Theory and Methods, N. U. Bang, F. K. Beller, E. Deutsch and E. F. Mammen (Eds), Academic Press, New York, 214). Platelet adhesion seems to correlate with the degree of fibrinogen adsorption to many materials (Lindon, J. N., McManama, G., Kushner, L., Kloczewiak, M., Hawiger, J., Merrill, E. W. and Salzman, E. W. (1987) In: Proteins at Interfaces: Physicochemical and Biochemical Studies, J. L. Brash and T. A. Horbett, (Eds), American Chemical Society, Washington, 507; Chaikof, E. L., Merrill, E. W., Coleman, J. E., Ramber, K., Connolly, R. J. and Callow, A. D., (1990) A. I. Ch. E. J. 36:994.). The complement system is activated by contact of blood with many types of hemodialysis membranes and oxygenators (Mollnes, T. E., Videm, V., Riesenfeld, J., Garred, P., Svennevig, J. L., Fosse, E., Hogasen, K. and Harboe, M. (1991) Clin. Exp. Immunol. 86, Suppl. 1:21).

Since these reactions are all manifestations of bio-incompatibility whose common thread is plasma protein interaction with the surface concerned, a natural approach to improving compatibility is to attempt to control the amount of relevant surface-associated protein. Insoluble or cross-linked polymers are the most prevalent type of biomaterial and a very wide variety of types have been tested for biocompatibility. However, the materials currently in use were not specifically designed for this purpose, rather they were tested because they were available for

other reasons (Ratner, B. D. (1993) J. Biomed. Materials Res. 27:837). As a result, a need remains for truly biocompatible materials, particularly where blood contacting applications are concerned.

The surface properties of materials polymerized in bulk are difficult to control due to the mobility of surface chains and the tendency of the surface material to adapt to the milieu in which it is located. The surface concentration of component parts of the polymers may not represent the bulk proportions and in extreme cases, such as with some polyetherurethanes (Lelah, M. D. and Cooper, S. L. (1986) Polyurethanes in Medicine, CRC Press, Boca Raton, FL.) local phase separation can occur.

One approach to providing a biocompatible surface with respect to protein adsorption and platelet adhesion has been to incorporate neutral polymers such as poly(ethylene glycol) (PEG; also known as poly(ethylene oxide) (Mori, Y., Nagaoka, S., Takiuchi, H., Kikuchi, T., Noguchi, N., Tanzawa, H. and Noishiki, Y., (1982) Trans. Am. Soc. Artif. Intern. Organs 28:459) or polyacrylamide (Fujimoto, K. *et al.* (1993) Biomaterials 14:442) into surface regions of solid polymers or hydrogels (Drumheller, P. D. and Hubbel, J. A. (1995) J. Biomed. Mater. Res. 29:207).

PEG may be incorporated into the polymer as a block, cross-linker or macromonomer (Drumheller, P. D. and Hubbel, J. A. (1995) J. Biomed. Mater. Res. 29:207; Brash, J. L. and Uniyal, S. (1979) J. Poymer Sci., Polymer Symp. 66:377; Sa Da Costa, V., Brier-Russell, D., Salzman, E. W. and Merrill, E. W. (1981) J. Coll. Interface Sci. 80:445; Takahara, A., Tashita, J., Kajiyama, T., Takayanagi, M. and MacKnight, W. J. (1985) Poymer 26:987) or grafted by reaction of gas phase monomers or oligomers with a substrate in a plasma discharge (D'Agostino, R. (Ed), Plasma Deposition, Treatment and Etching of Polymers, Academic Press, San Diego, (1990); López, G. P. *et al.* (1992) J. Biomed. Mater. Res. 26:415). In the above cases it is difficult to measure concentrations, molecular weights and dispositions of the

hydrated components at the surface although x-ray photoelectron spectroscopy (XPS) gives the elemental composition of the dry surface (Briggs, D. and Seah, M. P., Practical Surface Analysis by Auger and X-Ray Photoelectron Spectroscopy, Wiley, N.Y. (1983)).

It seems that PEG is somewhat incompatible with a wide variety of proteins so at sufficiently high PEG concentrations protein is excluded from the polymer coil. In the majority of cases reported (reviewed in Harris, J. M. (Ed), Biotechnical and Biomedical Applications of Poly(ethylene glycol) Chemistry, Plenum Press, New York, (1992)) both the amount of protein adsorbed and the adhesion of platelets in vitro has been reduced very significantly. Increasing PEG densities and molecular weights (up to 2,000) favour this reduction (Golander, C. and Kiss, E. (1987) J. Coll. Interface Sci. 121:240; Gambotz, W. R. (1988) Ph.D. Thesis, Centre for Bioengineering, University of Washington; Bergstrom, K., Holmberg, K., Safranj, A., Hoffman, A.S., Edgell, M. J., Kozlowski, A., Hovanes, B. A. and Harris, J. M. (1992) J. Biomed. Mat. Res. 26: 779). Protein adsorption is never eliminated, however; 5 - 10% or more of the control value remains (Golander, C. and Kiss, E. (1987) J. Coll. Interface Sci. 121:240; Gambotz, W. R. (1988) Ph.D. Thesis, Centre for Bioengineering, University of Washington; Bergstrom, K., Holmberg, K., Safranj, A., Hoffman, A.S., Edgell, M. J., Kozlowski, A., Hovanes, B. A. and Harris, J. M. (1992) J. Biomed. Mat. Res. 26: 779; Llanos, F. R. and Sefton, M. V. (1993) J. Biomed. Mater. Res. 27:1383).

Direct chemical grafting of preformed PEG to activated surfaces has been employed successfully in several studies (Harris, J. M. (Ed), Biotechnical and Biomedical Applications of Poly(ethylene glycol) Chemistry, Plenum Press, New York, (1992); Golander, C. and Kiss, E. (1987) J. Coll. Interface Sci. 121:240; Bergstrom, K., Holmberg, K., Safranj, A., Hoffman, A.S., Edgell, M. J., Kozlowski, A., Hovanes, B. A. and Harris, J. M. (1992) J. Biomed. Mat. Res. 26: 779; Tseng, Y.-C. and Park, K.

(1992) J. Biomed. Mater. Res. 26:373). Uniformity of coverage (detected by XPS) can be a problem however (Harris, J. M. (Ed), Biotechnical and Biomedical Applications of Poly(ethylene glycol) Chemistry, Plenum Press, New York, (1992)) and high density layers, in which the average chain separation is much less than the radius of gyration in solution, are not generally achieved. Presumably this is because in good solvents bound chains progressively exclude mobile chains as the layer density builds, reducing the probability of reaction between mobile chains and unreacted surface sites to negligible levels.

Hence, there remains a need for a biomolecule-exclusing polymer surface that is an efficient exclusion surface, for example a tethered layer in the "brush" configuration, that can be used as a biocompatible material or for size exclusion chromatography. There is a particular need for separation materials that can be used for analytical purposes of molecular weights up to 70,000.

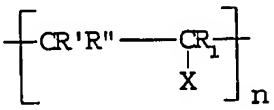
SUMMARY OF THE INVENTION

It is an object of this invention to provide a synthetic surface that excludes and/or minimizes the adsorption of proteins and other macromolecules. The approach taken works for a wide variety of macromolecules, not just proteins; e.g.: polysaccharides, nucleic acids, lipoproteins, synthetic polymers, etc. This surface has use as a size exclusion medium for chromatography and as a biocompatible material , because of the exclusion qualities of the relatively high polymer chain density.

It is another object of this invention to provide a method of synthesis for a high density tethered polymer surface comprising polymerizing vinyl monomers from an initial high surface concentration of initiating groups. The method of synthesizing this surface involves initiating polymerization of the surface polymer chains from the surface groups using CeIV or a metal carbonyl, which optimizes the density of chains

tethered to the surface.

It is a further object of this invention to provide a separating material comprising a non-porous substance coated with a co-polymer support having a substantially linear polymer covalently tethered thereto, said thether formed by polymerization of one or more types of vinyl groups via surface initiated polymerization of groups intrinsic to said co-polymer, said groups selected from the group consisting of aldehyde and sulfhydryl, wherein said linear polymer is selected from the group consisting of identical or different recurring units of formula (I)



(I)

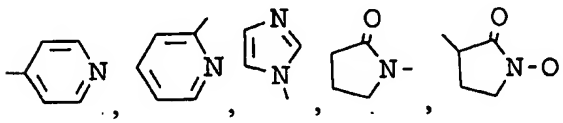
wherein

R_1 is H or Me;

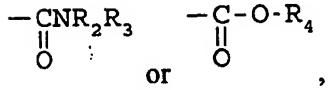
R' and R" are each independently H or CH_3,

X is $O=CO(CH_2CH_2O)_mH$, $O=CO(CH_2CH_2O)_mMe$, $O=CNH(CH_2CH_2O)_mH$, $O=CNH(CH_2CH_2O)_mMe$, $-C(O)NH_2$, $-C(O)OCH(OH)CH(OH)Me$, $-C(O)NCH_2CH_2OMe$, $-C(O)OCH_2CH_2OMe$, $-C(O)NCH(OH)CH(OH)Me$,

wherein m = 1 - 10,



, a polyethylene glycol,



and wherein

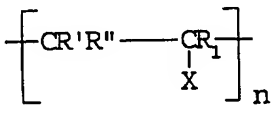
R_2 and R_3 are each independently:

(a) C_{1-10}-alkyl, phenyl, phenyl-C_{1-10}-alkyl, cycloalkyl, C_{1-10}-alkyl-cycloalkyl or C_{1-10}-alkylphenyl, wherein such compounds, contain one or more precursor groups for hydroxy or hydroxyl groups,

(b) a cyclic or bicyclic radical having 5-10 C atoms, wherein one or more CH or CH_2 groups is replaced by (i) N or NH, (ii) N or NH and S, or (iii) N or NH and O; and

n is 2 to 100.

It is yet a further object of this invention to provide a process for the prepartion of a material coated with a co-polymer support having a substantially linear polymer covalently tethered thereto which comprises the steps: (i) polymerizing selected monomers onto the material to form a co-polymer support, wherein at least one of said selected monomers has aldehyde or sulfhydryl groups, or is a precursor of said aldehyde or sulfhydryl groups; and (ii) grafting a tether to said co-polymer support by surface-induced polymerization of said co-polymer in the presence of a one or more groups of vinyl monomers to form a linear polymer tether, wherein said linear polymer tether formed is selected from the group consisting of identical or different recurring units of formula (I)



(I)

wherein

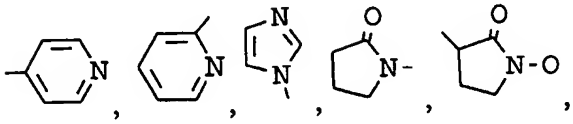
R_1 is H or Me;

R' and R" are each independently H or CH_3,

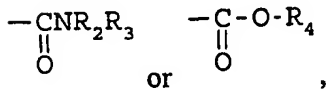
X is $O=CO(CH_2CH_2O)_mH$, $O=CO(CH_2CH_2O)_mMe$, $O=CNH(CH_2CH_2O)_mH$, $O=CNH(CH_2CH_2O)_mMe$, $-C(O)NH_2$, $-C(O)OCH(OH)CH(OH)Me$, $-C(O)NCH_2CH_2OMe$,

$-C(O)OCH_2CH_2OMe$, $-C(O)NCH(OH)CH(OH)Me$,

wherein m = 1 - 10,



, a polyethylene glycol,



and wherein

R_2 and R_3 are each independently:

(a) C_{1-10}-alkyl, phenyl, phenyl-C_{1-10}-alkyl, cycloalkyl, C_{1-10}-alkyl-cycloalkyl or C_{1-10}-alkylphenyl, wherein such compounds, contain one or more precursor groups for hydroxy or hydroxyl groups,

(b) a cyclic or bicyclic radical having 5-10 C atoms, wherein one or more CH or CH_2 groups is replaced by (i) N or NH, (ii) N or NH and S, or (iii) N or NH and O; and

n is 2 to 100.

DESCRIPTION OF THE FIGURES

Figure 1a: Conductometric titration of functional surface groups: Latex 14G1223 with HCl

Figure 1b: Conductometric titration of functional surface groups: Latex 14G1223 with NaOH

Figure 2: Proton NMR spectrum of latex A3

Figure 3: Conductometric titration for aldehyde content determination

Figure 4: Size distribution of Batch #14 seed

Figure 5: Size distribution of Batch #14G1

Figure 6: Size distribution of Batch #14G12

Figure 7: Size distribution of Batch #14G1233

Figure 8: Size exclusion of non-porous beads with grafted neutral polymer chains

Figure 9: An example of seed production and growing

Figure 10: An example of Acrolein derivatization

Figure 11: An example of grafting using Ce^{IV} on aldehyde group initiation

Figure 12: Comparative results of SEC on BIO SEC Merck gel and A10GR3 beads (7.5 cm x 0.47 xm, 0.1ml/min flow rate).

Table 1: Results of seeded Polymerization experiments

Table 2: Recipes for copolymerization styrene/acrolein on the latex

Table 3: Characterization of seed latexes

Table 4: First-stage growing latexes

Table 5: Second-stage growing latexes

Table 6: Third-stage growing latexes

Table 7: Surface charge density of the final latexes

Table 8: Aldehyde content of the derivatized latexes

Table 9: Complete example of parameter set.

Table 10: Optimum range of parameters

Table 11: Aldehyde derivatization

Table 12: Separation of proteins demonstrating charge neutrality of the polymeric tethers.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

A "biomaterial" may be defined as a material that is substantially insoluble in body fluids and that is designed and constructed to be placed in or onto the body or to

contact fluid of the body. Vascular grafts and contact lenses are examples of biomaterials.

As used herein, the solid surface of a biomaterial is characterized as "biocompatible" if it is capable of functioning or existing in contact with biological fluid and/or tissue of a living organism with a net beneficial effect on the living organism. Long term biocompatibility is desired for the purpose of reducing disturbance of the host organism.

Size exclusion chromatography is a chromatographic process in which the compounds in a mixture are separated on the basis of their hydrated size in solution, the larger molecules passing through the chromatography medium more rapidly than the smaller molecules, due to size-dependent exclusion from the stationary phase. Media which produce size exclusion chromatography exclude larger molecules to a greater degree than smaller molecules.

Grafting or surface-initiated polymerization is initiation of polymerization from a chemical group associated with the surface. The reaction adds monomers preferentially to the polymer chain attached to the surface as opposed to producing or adding to chains in solution that are not covalently attached to the surface. Two methods of initiation that produce such reactions are CeIV initiation and initiation by metal carbonyls in conjunction with UV radiation or heat.

The "brush" configuration of a tethered layer is defined as a layer of terminally grafted polymer with an average separation between grafted chains that is much less than the radius of gyration which the grafted chain would exhibit if it were not grafted but was free in solution experiencing no interactions with neighbouring chains (Fleer, F.J. *et al.*, *Polymers at Interfaces*, Chapman & Hall, 1993, Chapter 3).

A macromolecule is any oligomeric or polymeric material containing more than approximately ten monomers, or any non-polymeric species of molecular weight greater than approximately 500 g/mole. Examples are polypeptides, proteins, nucleic acids, polysaccharides, lipoproteins and synthetic polymers.

The Core Particle or Solid Surface

If the particle core or solid surface to be grafted is polymeric, it consists of precipitated or covalently cross-linked polymer that is insoluble in the solvent in which it is immersed and so produces a solid particle or other form of solid surface that does not contain pores. Examples are polystyrene, which is a precipitated polymer substantially insoluble in water or divinyl benzene-styrene copolymer, which forms covalently cross-linked particles that are substantially insoluble in all solvents that do not break covalent bonds. Many other polymeric particles and surfaces are known to those skilled in the art, including newly developed materials such as urea-melamine beads. In general, all types of polymers which may be produced in monodisperse beads in the size range between 0.1 to 50 microns may be used for both particles or solid surfaces.

If the particle or surface is not polymeric, it may be a solid, nonporous particle that has on its surface chemical groups that can be used for surface initiation or that may be chemically modified to provide such chemical groups. Alternately, it may act as a substrate for the adsorption of a polymer shell from which surface initiation may be performed. An example would be silica particles or glass surfaces that can be reacted with silane reagents to provide hydroxylated or aldehyde-containing surface groups. Alternately, hydrophobic silane reagents may be applied and a copolymer shell of styrene and acrolein adsorbed to the surface. In all these cases surface initiated polymerization could subsequently be carried out by, for instance, CeIV initiation. There is a large range of solid, non-porous particles and surfaces that could be used

for this purpose, as is well known to those skilled in the art.

The Polymer Shell

The polymer shell is typically a copolymer of (a) one monomer that is soluble in or adsorbs to the core particle or the solid polymer whose surface is to be grafted; (b) a monomer containing aldehyde or sulfhydryl groups from which grafting reactions may be initiated. In general, any chemical group carrying a hydrogen atom which may be oxidized can form a potential starting point for this process of surface polymerization. An example is a copolymer of styrene and acrolein that associates with the surface of polystyrene core latex particles or the surface of bulk polystyrene because of the solubility of styrene in polystyrene, and which provides aldehyde functions from which to initiate surface polymerization of vinyl monomers by CeIV initiation.

The copolymer may be crosslinked to inhibit it from dissolving during grafting by including a small percentage of divinyl benzene or some other suitable cross-linking agent, as is well known in the art of copolymer formulation.

The Tethered Polymers

The tethered polymer is a substantially linear polymer formed by the polymerization of one or more types of vinyl monomer via surface-initiated polymerization, typically by initiating polymerization with metal carbonyls and UV radiation or heat or with CeIV from aliphatic hydroxyl, aldehyde or sulfhydryl groups. For use in aqueous solutions, the vinyl monomers should be water soluble. For the synthesis of surfaces for size exclusion chromatography, the tethered polymers should be electrically neutral.

Modes of Synthesis

If the core particle or solid surface is a solid polymer latex, either emulsion polymerization or surfactant-free polymerization may be used for its synthesis. The shell copolymer typically is added by solution polymerization of a copolymer, one monomer of which was dissolved in the latex or adsorbed to its surface, usually using the same initiator as was used to synthesize the core latex. However, oil soluble initiators could also be used to advantage in certain systems. Those skilled in the art will recognize a number of well known ways by which to synthesize both the core and shell polymers.

The tethers are synthesized by use of a surface-initiated polymerization reaction, such as initiation with CeIV from aliphatic hydroxyl, aldehyde or sulfhydryl groups, or via metal carbonyl chemistry in combination with UV radiation or heat, utilizing water soluble vinyl monomers. Those skilled in the art will know a large number of suitable monomers, including (meth)acrylic acid derivatives such as acrylamide or methacrylamide, also 2,3-dihydroxypropyl methacrylate or N-(2-methoxyethyl) acrylamide or N-(2,3-dihydroxypropyl) acrylamide. Vinylated heterocyclic compounds may also be used to advantage, such as 1-vinylimidazole, N-vinylpyrrolidone, 2-vinylpyridine, 4-vinylpyridine and 4-vinylpyrrolidone-N-oxide. Macromers such as poly(ethylene glycol) methacrylates may also be used, which will produce tethers with a comb structure.

Moreover, a process recently developed for grafting polyacrylamide on polyethylene foils could be used. In this process, radicals are created on the foil using Co(60) radiation and the foil is immersed in the monomer solution.

Other Applications or Uses for Such a Molecule-Excluding Surface

As well as use as for size exclusion chromatography and in biomaterials, tethered polymer surfaces can be used to control the adsorption of molecules responsible for adhesion of microorganisms to surfaces so will be useful in producing anti-fouling surfaces. Moreover, depending on the type of polymer grafted onto a surface (ie. "compatible or incompatible" graftings), the polymer surface coating may be used to render surfaces sticky (ie. gluing together, or repulsing each other).

Advantages of This Invention

The grafting step of ths method provides more flexibility than traditional polymerized materials with regard to the density of the grafted tentacles and their length. Moreover, the density and length of the polymeric tethers can be customized to meet particular separation requirements.

In a preferred embodiment of the invention, the separation material will be best suited for analytical purposes of molecular weights up to 70,000. This is a distinct advantage over most gels, which show a poor separation capability at this size.

The material can also be optimized with respect to the diameter of the beads in order to obtain the maximum separation efficiency with the minimum back pressure.

The prior art contains polymer tethers grafted off primary and secondary hydroxyl groups. These types of surface initiation sites can not be used effectively for the separation materials of the present invention, however, because polymerization of -CH_2OH proceeds faster than via -CH_2COH because the free radical formed when the CH_2 is oxidized is mre stable for the aldehyde than the hydroxyl due to the intervening carbon. Hence, the polymerization reaction initiated from the aldehyde will proceed more slowly than in the cae of the hydroxyl group.

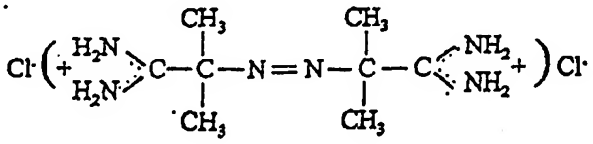
The rate of polymerization is important for obtaining an efficient exclusion surface, which is effectively provided by a tethered layer in the "brush" configuration, defined above.

This separation material will be mechanically stronger than traditional materials because of the lack of pores.

EXAMPLE I: SYNTHESIS OF A POLYMER SURFACE

Materials

All the distilled water used was further purified using a Milli-Q Plus water purification system. Styrene was Aldrich reagent grade material. It was purified by vacuum distillation at 40°C in an atmosphere of argon. Purified styrene was stored under argon at -70°C. Acrolein was Aldrich reagent grade material. It was purified by distillation at 56°C in an atmosphere of argon. Purified acrolein was stored protected from light, under argon at -70°C. 2,2'-Azobis (2-amidinopropane) dihydrochloride (ABA.2HCl) was supplied by Wako Co. and was used without further purification. The commercial name for the initiator is V-50 and the formula is the following:



Sodium chloride Fisher reagent grade was used without further purification.

Preparation of seed lattices

The apparatus consisted of a four-necked flask (1 dm^3 capacity), equipped with overhead stirrer, condenser, side-armed addition funnel, argon inlet with stopcock (argon outlet through the top of condenser). Argon flow was controlled by a needle valve and stirring rate by a tachometer. The flask was maintained at a constant

temperature, by immersion to the neck, in a thermostated water bath.

The following quantities of material were used:

0.72 dm^3	water
0.872 g	NaCl (2.07×10^{-2} moles/l)
0.54 g	ABA.2HCl (2.76×10^{-3} moles/l)
34.71 g	styrene (0.44 moles/l)

0.62 dm^3 water and the required amount of sodium chloride were initially placed in the flask, which was then evacuated eight times and flushed with argon under stirring (350 rpm).

The temperature of the bath was then increased to 70 ^{0}C, under stirring and slow argon flow initiated (1 bubble/s). Styrene was added under argon protection and then the funnel containing initiator dissolved in 0.1 dm^3 water, previously degassed and flushed with argon. After five minutes, initiator solution was released into the reaction mixture.

The reaction was allowed to proceed for 24 hours, at 70 ^{0}C, under argon flow, at 350 rpm stirring rate. The reaction mixture was then cooled at room temperature and filtered through glass wool to remove big aggregates. The product was cleaned by dialysis against distilled water for one week, in a 10 liter tank, changing the water every day. Further cleaning was done by centrifugation and washing at 2500 rpm. The latex suspension was then weighted and the solid content was determined by freeze drying. The yield was calculated, relative to the total amount of styrene introduced.

All the latex suspensions, after cleaning, were stored at 4 ^{0}C, in polypropylene tubes, until future use.

Seeded polymerizations

A number of experiments were conducted in order to optimize the conditions for obtaining reasonably monodisperse polystyrene beads, with a size in the range 1-3 μm, using ABA.2HCl as initiator.

A reaction vessel (as described in Section 6.1.1), having a capacity of either 0.25 l or 0.5 l was used. The seed latex was first weighed and put in the flask and the desired concentration of solids was adjusted using distilled water. Sodium chloride was used in some experiments to achieve a desired ionic strength. The apparatus was evacuated eight times and flushed with argon under stirring at 350 rpm, then the temperature was increased to the desired value. Styrene was added under argon protection. The seed latex was swollen under the same stirring regime and argon flow (1 bubble/s).

Initiator was then added and the reaction continued for the prescribed time. The product was treated exactly as described above. In some reactions, because the product was aggregated, it was sonicated for 30 min. before filtering through the glass wool. To prevent further aggregation, the sonicating bath was cooled with ice.

Grown latexes were characterized with respect to size distribution and solid content. The yield was calculated for the growth reactions.

Detailed recipes for these steps are written in TABLE 1.

In all experiments except for 10G12 (100 g/l), the concentration of styrene relative to total aqueous phase was 80 g/l.

Surface aldehyde derivatization

The work described in the literature involving polystyrene latex with aldehyde groups on the surface refers to much smaller beads, produced directly by polymerization of a mixture of the two monomers utilizing potassium persulphate as initiator. The present invention also involves a procedure for derivatization of previously grown beads. A mixture of styrene and acrolein was used to produce a shell around the polystyrene core.

The monomer ratio was calculated according to the copolymerization curve (*Polymer Handbook*, J. Brandrup and E.H. Immergut, eds, Section II:110, InterScience Publications, N.Y., 1966) to give azeotropic conditions (*i.e.*, composition of the feed equal to the composition of the resulting copolymer). The experimental setup was the same as above. The procedure was as follows:

Seed latex was charged in the flask, which was then evacuated and flushed with argon eight times. The temperature was raised to 50 ^{0}C, under gentle argon flow and stirring at 350 rpm. Styrene was added and the seed allowed to swell for 15 min., then acrolein dissolved in 10 ml water was put into the reaction vessel, followed by initiator solution (in 10 ml water, washed in with 10 ml more). The reaction was continued at 50 ^{0}C, under argon flow (one bubble/s) and with stirring at 350 rpm for 6 h. (See Table 2)

Analytical methods

The size distribution was determined from scanning Electron Microscopy images of the latex (one drop was dried on carbon plate, then covered with gold). An image analysis program was used to measure the diameters of at least 100 beads on several pictures taken at different spots on the plate.

Surface Charge Density

The surface functional groups on the beads are initiator residues only, because the method used to synthesize them was emulsifier-free. They consist of amidine groups, positively charged. The suspension stability of the latex results from the presence of these groups on the surface of the microspheres, hence the surface charge density (*i.e.* concentration of surface functional groups) is an important characteristic of the product.

A conductometric titration technique was used to determine surface charge density of the latexes used for aldehyde derivatization. The method is described in the literature. The only modification was that the cleaning step to remove detergent, involving ion exchange resins was omitted, because the beads were surfactant-free. The samples (already dialysed, as described above) were further prepared for titration only by washing once in water (centrifugation at 1500 rpm, removal of supernatant and replacement with fresh distilled water). The solid content was determined by freeze drying and weighing the initial suspension and solid residue. At least 0.5 g of solid latex was then suspended in 10 ml of water, purged with argon for 5 minutes and placed in the conductometric cell under slow argon flow. A conductivity meter was used to monitor conductance, while 0.01 M titrant (either HCl or NaOH) was added to the sample, using a precision pump, under vigorous stirring. Titrations were performed at constant flow rate (0.0204 ml/min.) with time monitoring. For each sample, the equivalence point was found twice, once from direct titration using HCl and the second time from backwards titration using NaOH. The results are reported as the average for two samples.

Both solutions used for titration were standardized by potentiometric titration. NaOH was first used to titrate a standard solution of potassium biphtalate, then HCl was used to titrate the NaOH solution of known concentration.

Aldehyde content analysis

Nuclear Magnetic Resonance

For the latexes with high aldehyde surface concentration, proton Nuclear Magnetic Resonance (NMR) spectroscopy was used to determine the aldehyde content. The samples for NMR were prepared as follows:

The latex suspension containing approximately 30 mg solids was freeze dried for 24 hours, then the solid residue dissolved in 1 ml deuterated tetrahydrofuran. Traces of water were removed from the solution by keeping it in contact with molecular sieves overnight. The sample was then transferred into an NMR tube, previously flushed with argon.
A Brucker 400 Mhz spectrometer was used to record the spectrum.

Conductometric Titration

For latexes with low surface aldehyde concentration, NMR was not sensitive enough to provide a reliable assay. Instead, a method from the literature for determining aldehyde content based on conductometric titration was used. This involves reacting the aldehyde groups with hydroxylamine hydrochloride:

$$NH_2OH.HCl + R\text{-}HC{=}O \qquad HCl + R\text{-}CH{=}N\text{-}OH + H_2O + HCl$$

The hydrochloric acid resulting is titrated conductometrically with sodium hydroxide.

The experimental procedure is described below:

A 0.1M solution of hydroxylamine hydrochloride was prepared in a volumetric flask (M.W.= 69.5; 0.695g/100mL). An exact volume of this solution was added to a concentrated suspension of latex of known solid content. The mixture was left to react overnight while tumbling in a rotating rack at room temperature. The suspension was then filtered through a membrane filter (0.22 µm pore size), and diluted to 10 mL, then transferred to the conductometric cell. The titration proceeded under argon. Sodium hydroxide solution, previously standardized, was added from a glass syringe using a precision pump. During the titration, the time and the conductance were monitored, at constant flow rate and the time converted to volume. The equivalence point was read on the conductance versus titrant volume plot. The result reported is the average of two titrations.

EXAMPLE 2: PRODUCTION OF SEED LATEX

Grafting on latex A8 is accomplished as follows. The reactor is a three-necked vial (capacity 50 ml), equipped with argon inlet and outlet (with stopcocks).

Latex suspension (2 g solids suspended in 9 ml water) and MEA monomer (0.92 g) are introduced in the reactor, which is then degassed two times and flushed with argon. The initiator solution (0.31 g cerium (IV) ammonium nitrate is dissolved in 2 ml of 10 mM nitric acid solution) is added from a syringe, which is previously degassed. The content of the vial is mixed by hand, then stirred for one hour at 40 °C. The reaction is then continued for 64 hours at room temperature.

The product is suspended in 250 ml water, filtered on Millipore membrane (1.25 µm), washed on the filter with 25 ml 0.1 M sodium sulfite solution and then with 50 ml 0.03 M EDTA (ethylenediaminetetraacetic acid trisodium salt hydrate) solution and rewashed with water. The product is resuspended in 25 ml water and stored in

polypropylene tubes at 4 °C until further use.

EXAMPLE 3: CHARACTERIZATION OF THE LATEX

Typical values for the characteristics of the seed latex produced by the described technique are given in Table 3. Typical values for the conditions that lead to successful first, second and third growth stages are summarized in Table 9. In this Table, latexes 12G1 and 14G1 have gone through one growth stage, latexes 12G12 and 14G12 have completed the second growth stage and 12G1233 and 14G1233 have gone through the third growth stage. For each the important parameters describing the synthetic conditions that gave stable, uniform latex preparations are listed. The optimum range these synthesis parameters could take and still produce acceptable products are summarized in Table 10. Table 11 presents two examples of synthesis of the shell copolymer added to the two parents described in Table 9 where the critical parameters are listed that again produced stable products. The optimum range for the shell synthesis parameters are as follows:

1. Acrolein to seed ratio: 1 x 10^{-3} to 2 x10^{-3} moles per gram latex
2. Initiator concentration: 4.23 x10^{-4} to 4.4 x10^{-3} moles dm^{-3}
3. Charge density of seed: 154 $Å^2$/group to 314 $Å^2$/group

The physical properties of typical latexes at each stage of the growth process are given in Figures 4-7 (size distribution) and Table 7 (surface charge density) and Table 8 (aldehyde content by two methods).

A grafted layer of N-(2- methoxyethyl) acrylamide (MEA) was added to the core/shell latex A8, as described above. The amount of monomer grafted was determined from analysis of the amount left in solution following polymerization.

The method was based on the behaviour of the monomer on HPLC on RP Select B (Merck), 10 diameter beads, 4.5 cm x 1.0 cm diam. column, 3ml/min flow rate, in a gradient of 0.01 M tetrafluoroacetic acid (TFA) and 50% acetonitrile in 0.01M TFA. The monomer concentration was determined by the optical density (OD) at 240 nm. The column was calibrated with pure monomer of known concentration. The amount grafted was found to be 3.56×10^{-3} mole/g latex.

The grafted beads were packed into a column 0.46 cm diam x 7.5 cm and a series of proteins of known molecular weight, dissolved in a buffer of 10 mM phosphate, 300 mM NaCl, pH 7.2, at a concentration of approximately 0.5 mg/ml were chromatographed. The flow rate was 0.01 ml/min and the OD of the eluent stream was monitored at 280 nm. The results are shown in Figure 8 in which it is seen that good separation on the basis of molecular weight is obtained.

Hence, size exclusion chromatography of proteins is demonstrated on solid polystyrene beads carrying neutral polymer tethers.

EXAMPLE IV: DEMONSTRATION THAT POLYMERIC TENTACLES ARE NEUTRALLY CHARGED

The following was conducted to demonstrate that, despite the fact that the core beads are positively charged, after grafting the charge is completely shielded by the neutral tentacles. The demonstration was performed using a 7.5 x 0.47 cm column packed with beads. Proteins were fun at 0.1 ml/min, using high ionic strength buffer (10 mM NaH_2PO4, 300 mM NaCl, pH 7.2) and then low ionic strength buffer (2.5 mM NaH_2PO_4, 2.5 mM NaCl, pH 7.2). The elution times are shown in Table 12.

TABLE 12

Sample	Molecular Wt g/mole	Elution time, min High ionic strength mobile phase	Elution time, min Low ionic strength mobile phase
$NaNO_3$	69	5.70	5.74
Insulin	6,500	5.66	5.24
Myoglobin	17,200	5.23	5.18
Trypsin Inhibitor	21,500	5.10	5.14
Ovalbumin	43,000	5.09	5.07
Keyhole Limpit Hemocyanin	~3,000,000	4.84	4.53; 5.22 (two peaks)

EXAMPLE V: COMPARISON STUDY

A comparison between various materials available on the market was published in a theoretical paper (see Brooks and Muller, 1996). The results show that the BIO SEC gel (Merck) gives the best performance in terms of providing the highest selectivity, as reflected by the highest slope on the graph displayed in Fig 1., p. 699 of the publication. It also demonstrated the highest exclusion effect, as shown by the highest absolute value of the intercept.

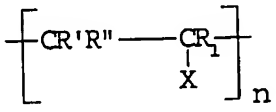
The size exclusion performance of the separation material of the present invention, in a bead embodiment, was compared with the highest performing BIO SEC gel. The A10GR3 beads were used for comparison. The proteins used to demonstrate were: insulin 6,500; myoglobin 17,200; trypsin inhibitor 21,500; ovalbumin 43,000; bovine serum albumin 68,000; immunoglobulin G 156,000. The proteins were

chromatographed under identical conditions on a column 7.5 cm x 0.47 cm.; 0.3 M NaCl, 10 mM phosphate, pH 7.2, at a flow rate of 0.1 ml/min. The back pressure at this flow rate was 3 bars on the BIO SEC gel column and 20 bars on A10GR3 column.

The results are presented in Figure 12. The parameters of the lines were: BIO SEC slope = -3.2×10^{-6}, intercept = -0.222; for A10GR3, the slope = -2.2×10^{-6}, intercept =0.324. The results show lower non-specific adsorption on A10GR3 beads (higher absolute value of the intercept) and better linear dependence in the low molecular weight region. Also, the values of all the elution times were lower on the beads (separation was faster, no time consumed for diffusion in and out of the pores).

We claim:

1. A separating material comprising a non-porous substance coated with a co-polymer support having a substantially linear polymer covalently tethered thereto, said thether formed by polymerization of one or more types of vinyl groups via surface initiated polymerization of groups intrinsic to said co-polymer, said groups selected from the group consisting of aldehyde and sulfhydryl, wherein said linear polymer is selected from the group consisting of identical or different recurring units of formula (I)



(I)

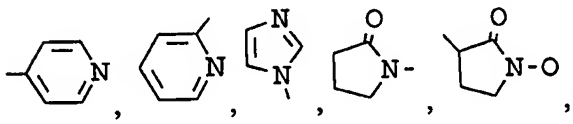
wherein

R_1 is H or Me;

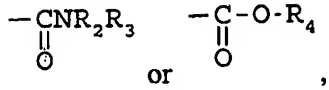
R' and R" are each independently H or CH_3,

X is $O=CO(CH_2CH_2O)_mH$, $O=CO(CH_2CH_2O)_mMe$, $O=CNH(CH_2CH_2O)_mH$, $O=CNH(CH_2CH_2O)_mMe$, $-C(O)NH_2$, $-C(O)OCH(OH)CH(OH)Me$, $-C(O)NCH_2CH_2OMe$, $-C(O)OCH_2CH_2OMe$, $-C(O)NCH(OH)CH(OH)Me$,

wherein m = 1 - 10,



, a polyethylene glycol,



and wherein

R_2 and R_3 are each independently:

(a) C_{1-10}-alkyl, phenyl, phenyl-C_{1-10}-alkyl, cycloalkyl, C_{1-10}-alkyl-cycloalkyl or C_{1-10}-alkylphenyl, wherein such compounds, contain one or more precursor groups for hydroxy or hydroxyl groups

(b) a cyclic or bicyclic radical having 5-10 C atoms, wherein one or more CH or CH_2 groups is replaced by (i) N or NH, (ii) N or NH and S, or (iii) N or NH and O; and n is 2 to 100.

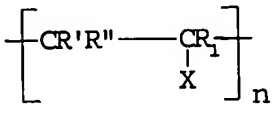
2. The separating material as in claim 1, wherein n is 20 to 50.

3. The separating material as in claim 1, wherein n is about 40 to 50.

4. A process for the preparation of a material coated with a co-polymer support having a substantially linear polymer covalently tethered thereto which comprises the steps:

(i) polymerizing selected monomers onto the support to form a co-polymer support, wherein at least one of said selected monomers has aldehyde or sulfhydryl groups, or is a precursor of said aldehyde or sulfhydryl groups; and

(ii) grafting a tether to said co-polymer support by surface-induced polymerization of said co-polymer in the presence of a one or more groups of vinyl monomers to form a linear polymer tether, wherein said linear polymer tether formed is selected from the group consisting of identical or different recurring units of formula (I)



(I)

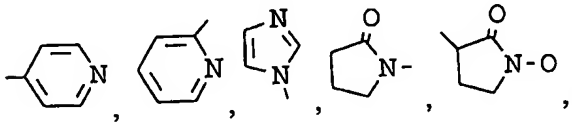
wherein

R_1 is H or Me;

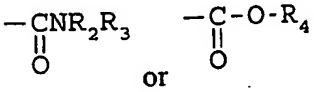
R' and R" are each independently H or CH_3,

X is $O=CO(CH_2CH_2O)_mH$, $O=CO(CH_2CH_2O)_mMe$, $O=CNH(CH_2CH_2O)_mH$, $O=CNH(CH_2CH_2O)_mMe$, $-C(O)NH_2$, $-C(O)OCH(OH)CH(OH)Me$, $-C(O)NCH_2CH_2OMe$, $-C(O)OCH_2CH_2OMe$, $-C(O)NCH(OH)CH(OH)Me$,

wherein m = 1 - 10,



, a polyethylene glycol,



and wherein

R_2 and R_3 are each independently:

(a) C_{1-10}-alkyl, phenyl, phenyl-C_{1-10}-alkyl, cycloalkyl, C_{1-10}-alkyl-cycloalkyl or C_{1-10}-alkylphenyl, wherein such compounds, contain one or more precursor groups for hydroxy or hydroxyl groups,

(b) a cyclic or bicyclic radical having 5-10 C atoms, wherein one or more CH or CH_2 groups is replaced by (i) N or NH, (ii) N or NH and S, or (iii) N or NH and O; and

n is 2 to 100.

5. A separating material produced by the method of claim 4.

6. A biocompatible surface produced by the method of claim 4.

7. An antithrombotic surface produced by the method of claim 4.

Figure 1a

Titration of latex 14G1223 (0.776g) using HCl 0.0118M

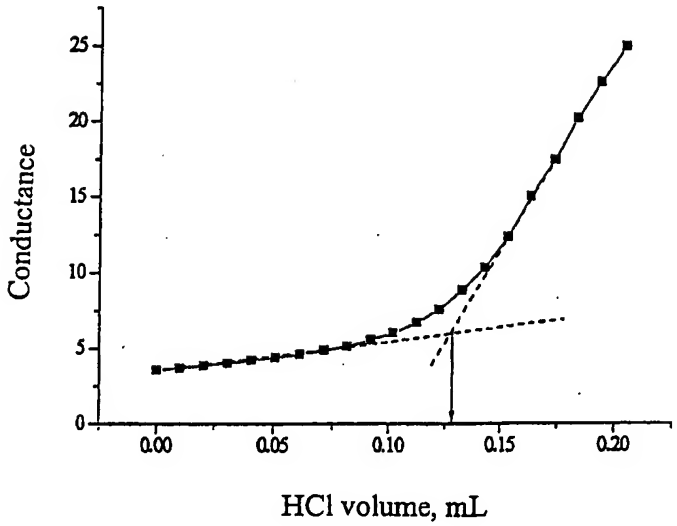


Figure 1b

Titration of latex 14G1223 (0.776g, 0.204 ml HCl 0.0118M added) using NaOH 0.00933M

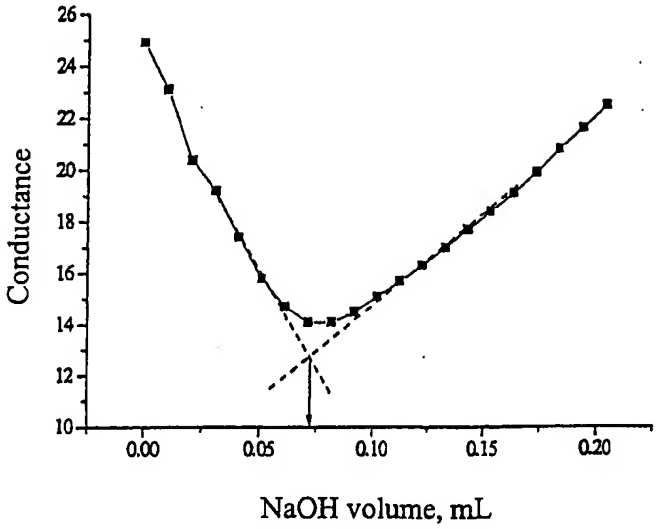


Figure 2

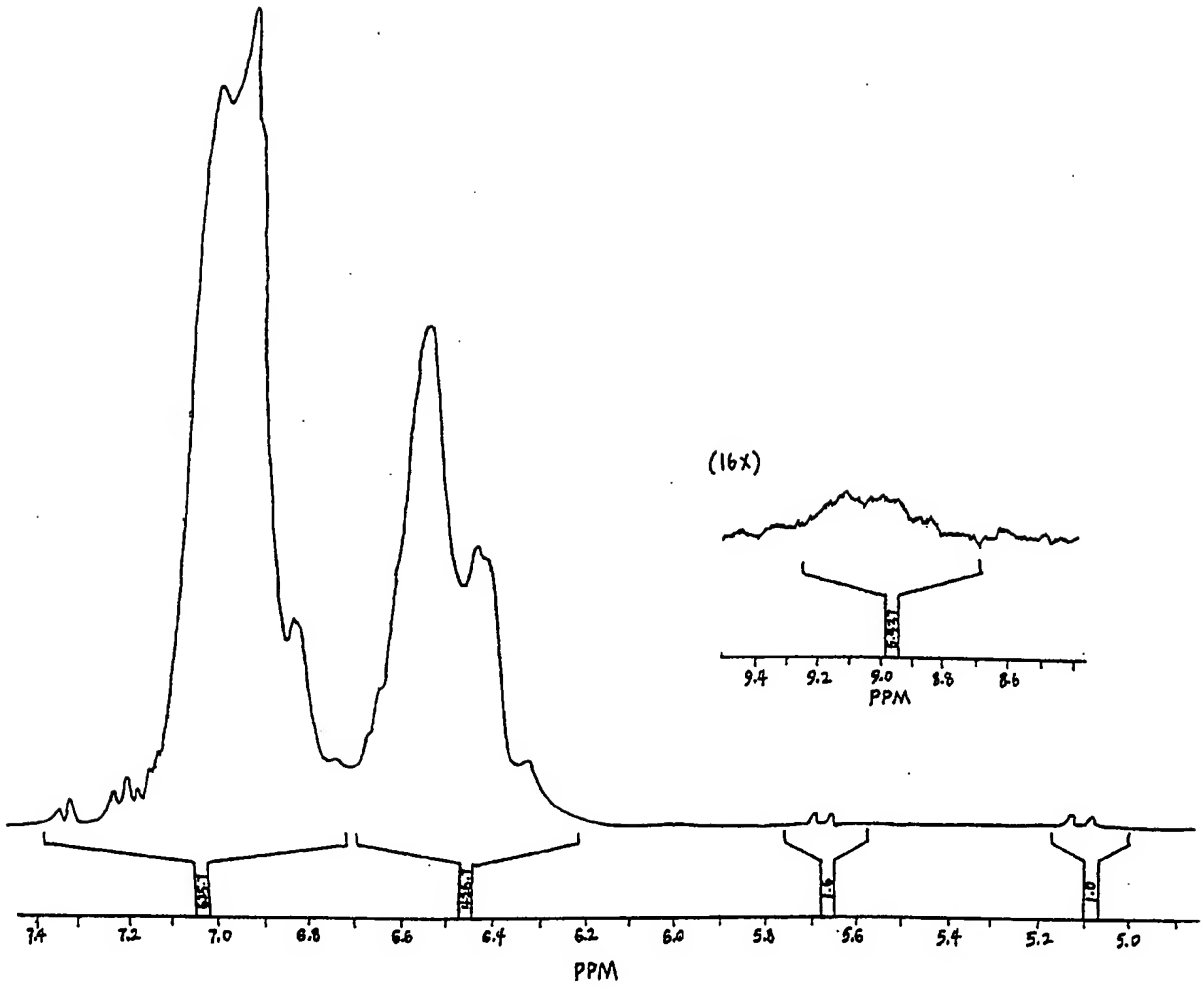


Figure 3

Titration of latex A10 (0.561g, 1.2×10^{-5} moles hydroxylamine added)

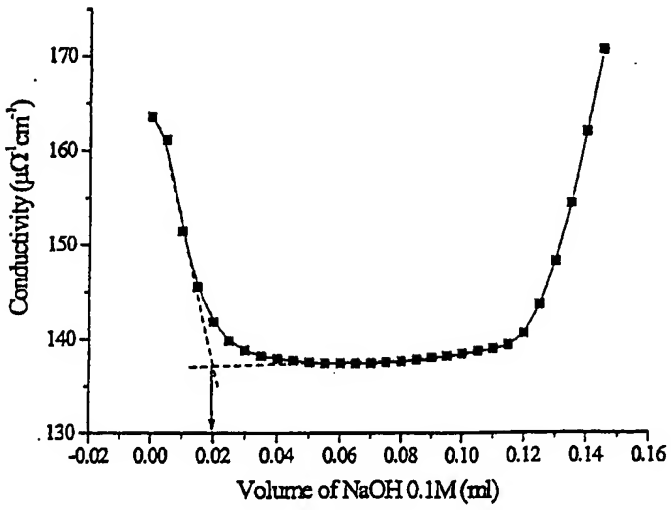


Figure 4

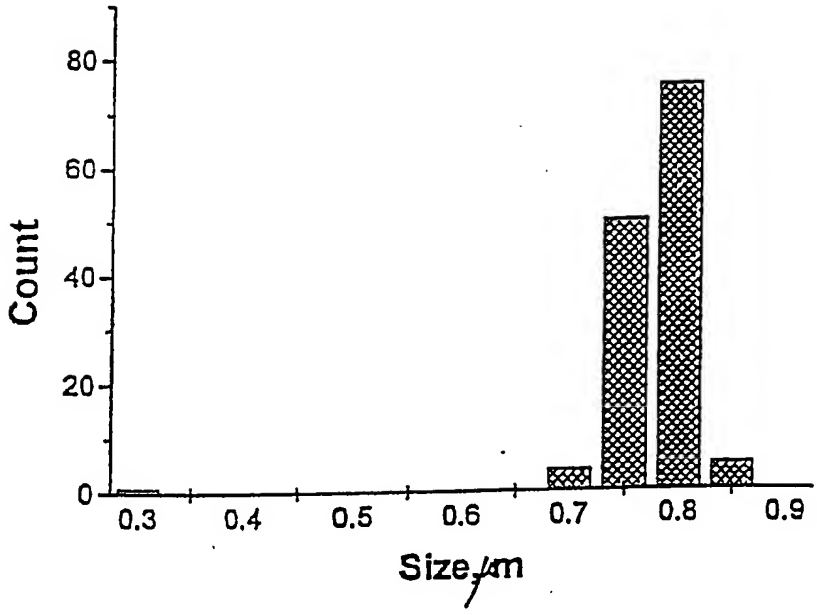


Figure 5

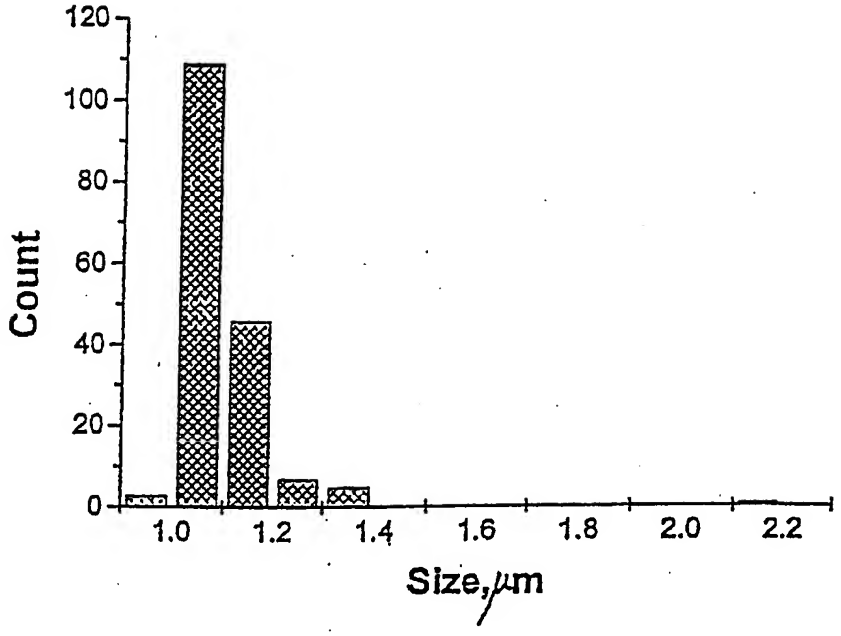


Figure 6

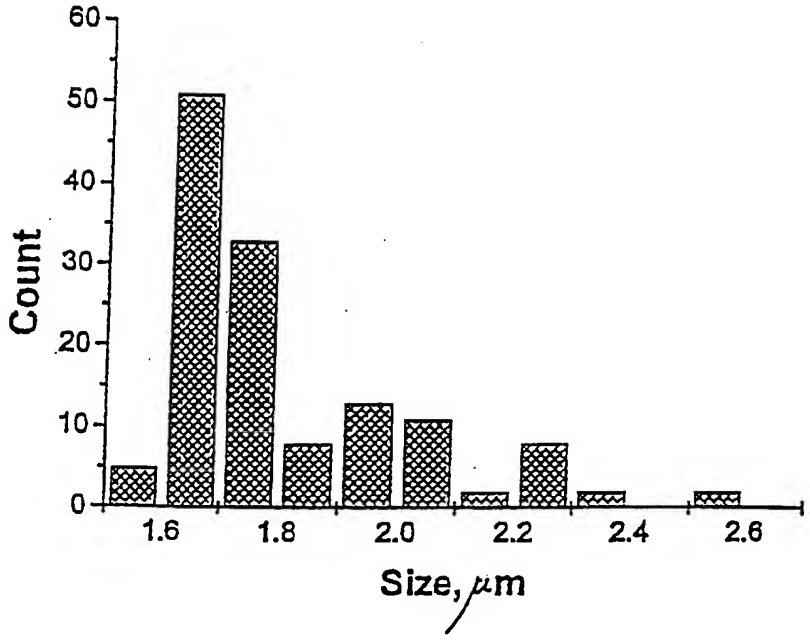
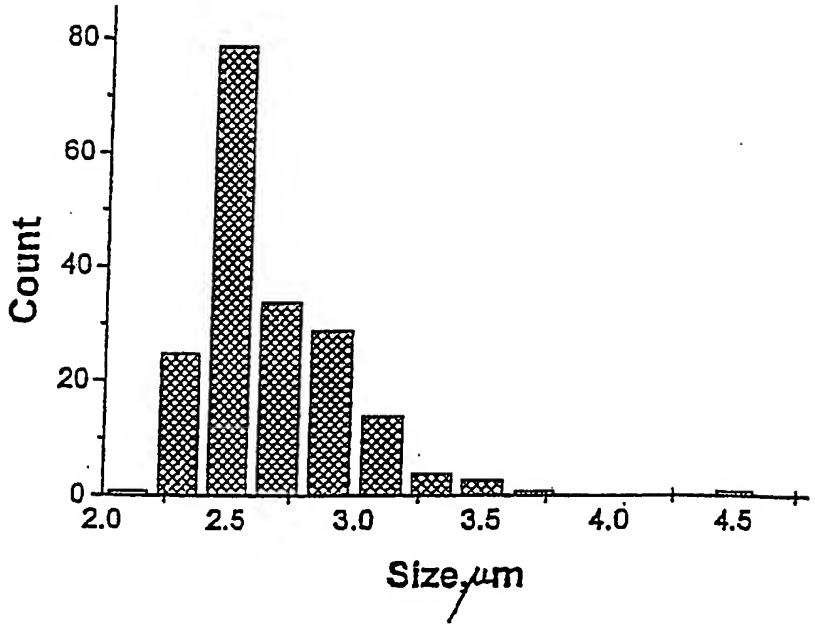


Figure 7



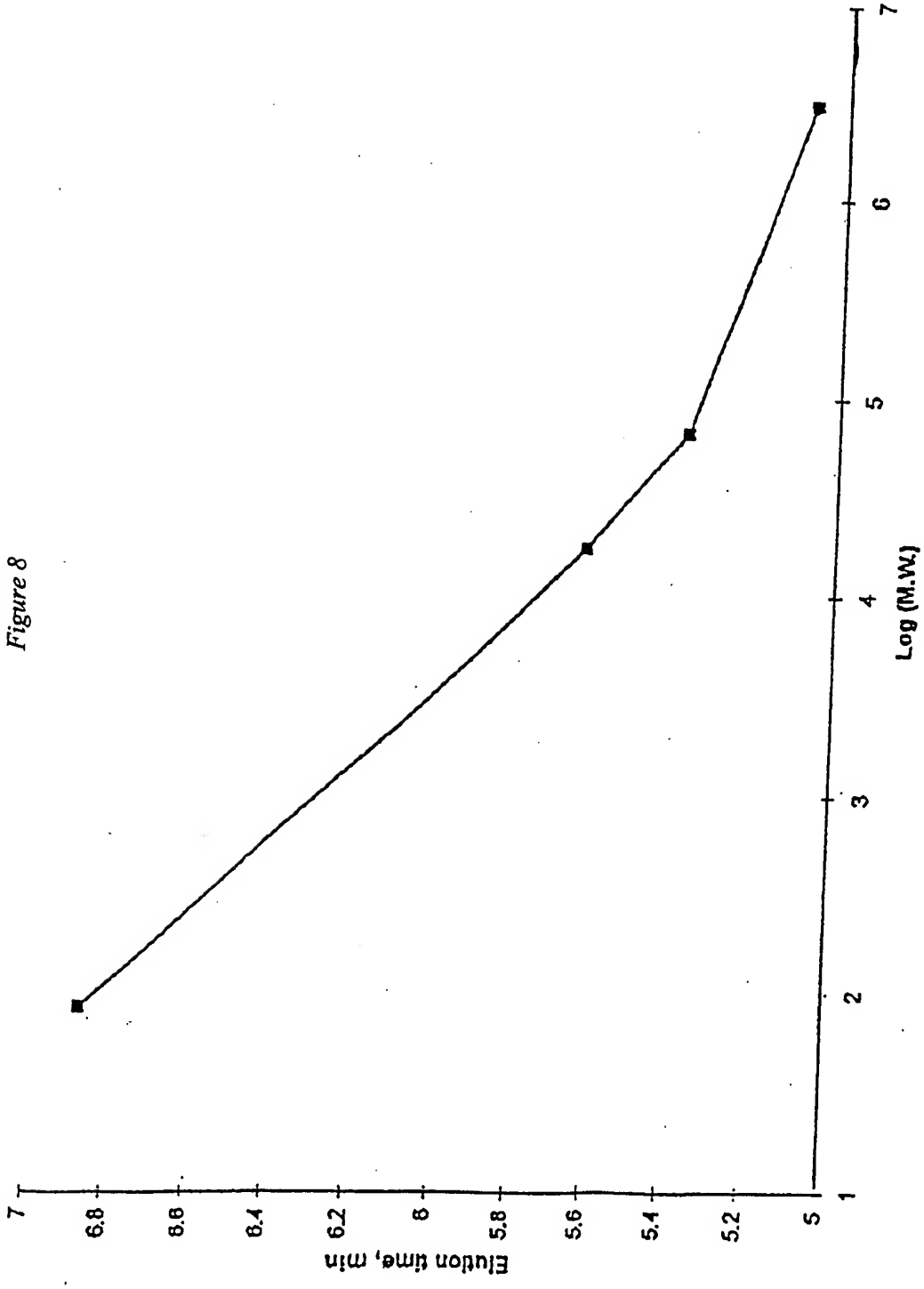
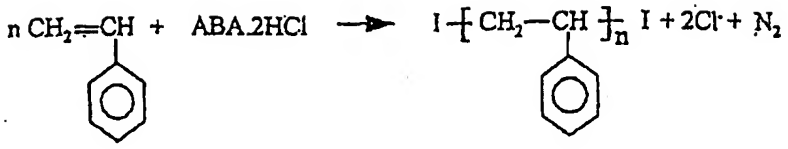
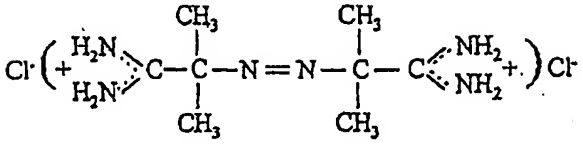


Figure 8

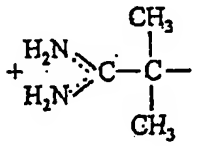
Figure 9



where ABA.2HCl is:



I - is the initiator residue on the surface of the bead:



Structure of the cationic polystyrene bead after growing steps is:

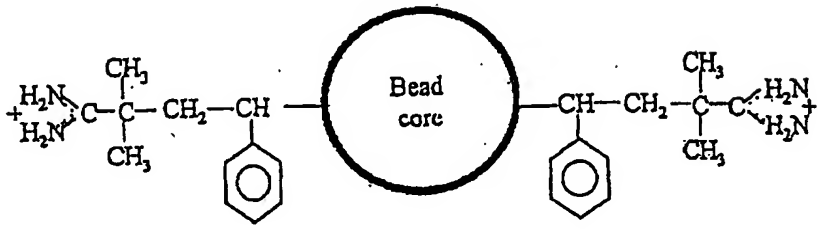
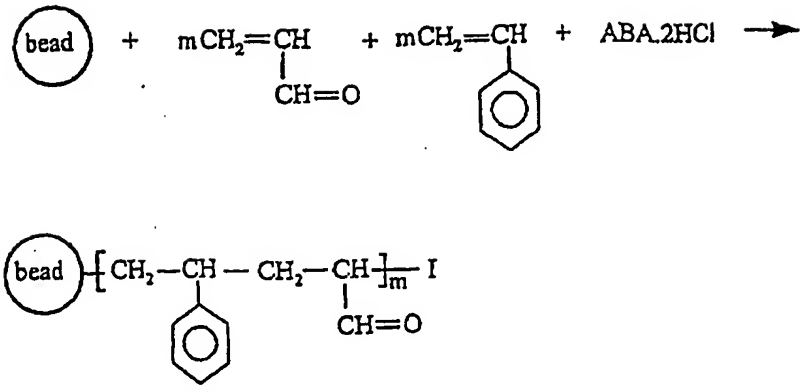


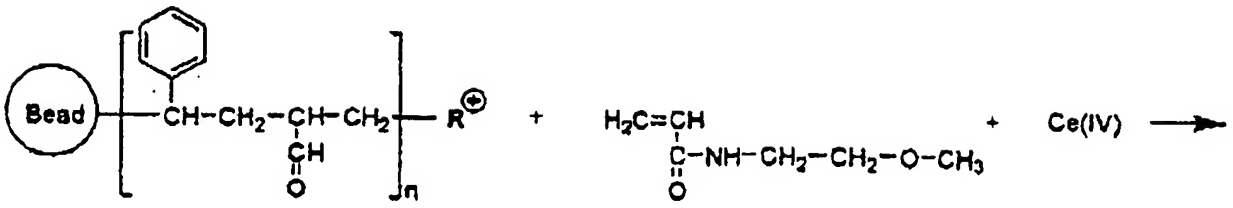
Figure 10



I- is the same as before.

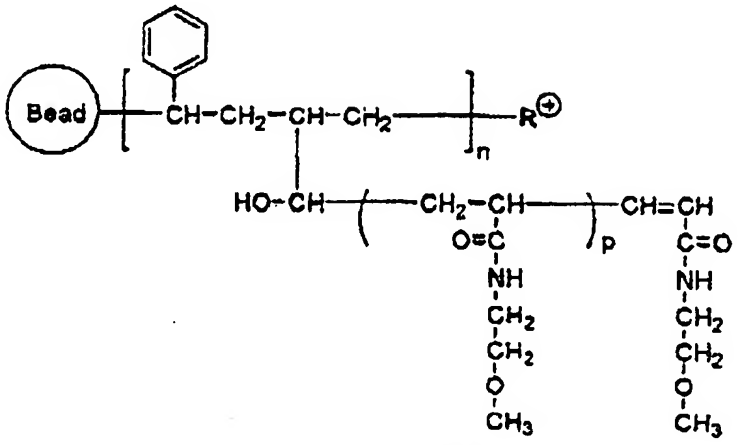
The copolymer shell on the surface of the bead has proably an alternating structure.

Figure 11



Positively charged bead with poly(styrene-co-acrolein) shell

N(2-methoxy ethyl) acrylamide



Bead bearing grafted chains

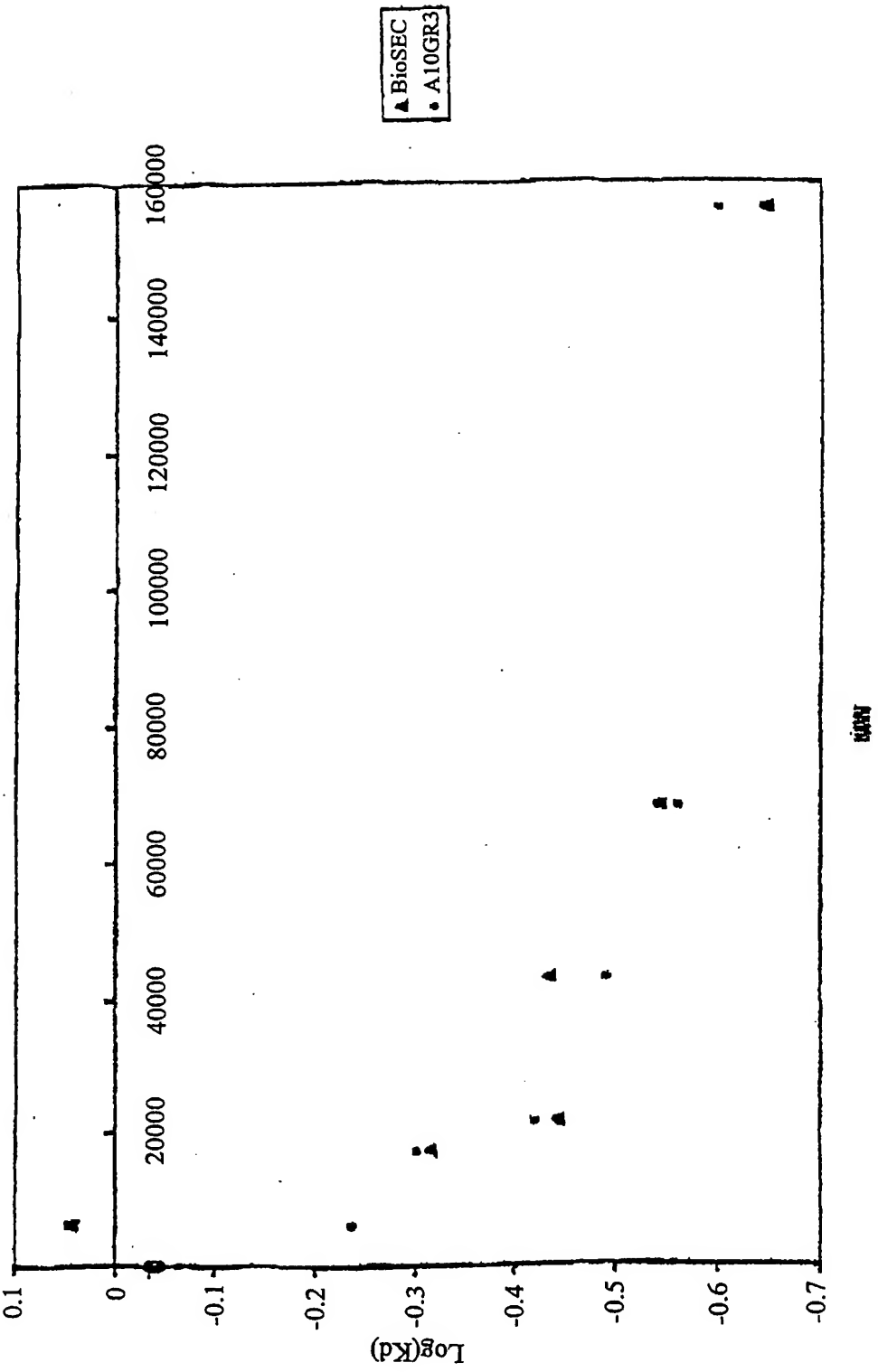

BioSEC
A10GR3
160000
140000
120000
100000
80000
60000
40000
20000
0
0.1
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
Log(Kd)

Figure 12

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/IB 98/00563

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 B01J20/32 G01N30/48 A61L33/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 B01J G01N A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 453 186 A (W. MÜLLER) 26 September 1995 cited in the application see the whole document ---	1-7
A	EP 0 106 769 A (COMMISSARIAT A L'ENERGIE ATOMIQUE) 25 April 1984 see page 15; example 11 see page 8, line 31 - page 9, line 2 see page 4, line 9 - line 22 -----	1-7

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 August 1998	26/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hilgenga, K

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/IB 98/00563

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US 5453186 A	26-09-1995	DE 3811042 A	19-10-1989
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		CN 1036516 A,B	25-10-1989
		CS 8901981 A	19-02-1992
		EP 0337144 A	18-10-1989
		JP 1310744 A	14-12-1989
EP 106769 A	25-04-1984	FR 2534486 A	20-04-1984
		CA 1223859 A	07-07-1987
		JP 59192958 A	01-11-1984